Volume 324, number 2, 237–240 FEBS 12593 June 1993

Thermal transitions in the purple membrane from *Halobacterium halobium*

Valery L. Shnyrov and Pedro L. Mateo

Departamento de Quimica Fisica, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain

Received 23 April 1993

The application of a successive annealing procedure to the scanning calorimetric endotherm of the purple membrane from *Halobacterium halobium* in phosphate buffer, pH 7.5, leads to five thermal transitions beneath the overall endotherm. Circular dichroism and fluorescence experiments have also been carried out with the native membrane heated at the same scan rate as in calorimetric runs (1°C/min) as well as with previously heated membrane samples. These results, together with others from the literature, have been used to suggest a preliminary explanation of the five thermal transitions.

Bacteriorhodopsin, Purple membrane; Thermal transition; Scanning calorimetry; Circular dichroism; Fluorescence

1. INTRODUCTION

The purple membrane (PM) of the halophilic bacteria *Halobacterium halobium* contains a single protein, the light-driven proton pump bacteriorhodopsin (BR), which is organized in a two-dimensional, hexagonal lattice of protein trimers (for reviews, see [1,2]). BR is an integral membrane protein composed of seven transmembrane helices connected by short extramembranous loops [3]. Lipids are present in remarkably low amounts (approximately 75% protein and 25% lipid) and have a very unusual composition [4]. All this goes to make PM a most appropriate native-membrane system on which to attempt a thermodynamic analysis [5–16]. Nevertheless, the analysis of the complex thermal profile of PM has only led so far to the description of the predenaturational and main transitions under the overall differential-scanning-calorimetric (DSC) endotherm. The aim of this report is to describe further advances with this type of analysis, using different techniques to follow the temperature-induced processes in PM.

2. MATERIALS AND METHODS

PM was isolated from *Halobacterium halobium* strains P-353 and S9 as described [17]. The concentration of BR was determined spectrophotometrically by using $\varepsilon_{568} = 63{,}000\ M^{-1} \cdot cm^{-1}$ [18]. Protein concentrations were $1–2 \times 10^{-4}$ M in calorimetric experiments, $5–10 \times 10^{-6}$ M in fluorometric analysis and 10^{-6} M in circular dichroism (CD) measurements.

DSC experiments were performed with a high-sensitivity differential adiabatic scanning microcalorimeter DASM-4 [19] with cell volumes of 0.48 ml, at a heating rate of 1°C/min. An overpressure of 2 atm was always kept over the liquids in the cells throughout the scans to prevent any degassing during heating.

Fluorescence spectra were recorded with a MPF-4B spectrofluorimeter (Perkin-Elmer). All fluorescence spectra were corrected for the instrumental spectral sensitivity. Protein fluorescence quantum yield was evaluated by comparing areas under fluorescence spectra of the protein sample with that of an aqueous tryptophan solution [20] with the same absorbance at the excitation wavelength of 280 nm. The position of the middle of a chord drawn at the 80% level of the maximum intensity λ_{max} was taken as the position of the spectrum. The temperature of the sample compartment was raised at a constant rate of about 1°C/min using thermostatically controlled water circulating in a hollow, brass cell holder. The temperature in the sample cell was monitored by means of a copper-constantan thermocouple.

CD spectra were recorded with a Jasco J-500A spectropolarimeter in a 0.237 mm cell jacketed in a hollow, brass cell holder connected to a thermostatically controlled waterbath at 20 nm/min with a sensitivity range of 0.5 mdeg/cm. The temperature of the sample compartment was raised at a constant rate of about 1°C/min. The cell temperature was measured with a thermistor.

3. RESULTS

Fig. 1A shows a typical DSC scan for a PM suspension in sodium phosphate, pH 7.5. Researchers have generally taken into account only two transitions, the so-called pre-transition, at about 75°C (reversible), and the main transition (irreversible), centered at about 95°C, claiming that the main transition corresponds to BR denaturation [5] and that "the denaturational transition is distinctly asymmetrical, and often a shoulder is discernable on the low-temperature side" [9]. We show here the rather complicated nature of the PM thermal absorption, using a previously described successive annealing procedure [21], which has been applied to several proteins to analyze their DSC profiles [22–24].

Correspondence and present address: V.L. Shnyrov, Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Salamanca, Plaza de la Merced, 1-5, 37008 Salamanca, Spain. Fax: (34) (23) 294513.

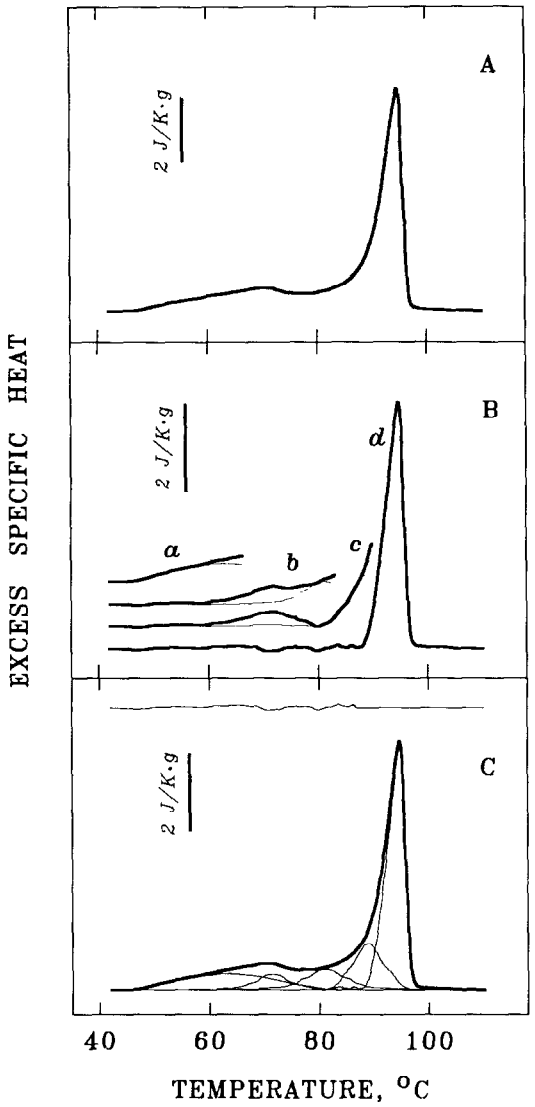

Fig. 1. (A) Calorimetric profiles of PM suspension in 0.19 M phosphate, pH 7.5, at the scanning rate 1°C/min. (B) Demonstration of the successive annealing procedure for the PM suspension. The curves are displaced along the ordinate for clarity. (C) Results of the deconvolution of the heat-capacity curve into individual components by the successive annealing procedure. Subtracting the sum of the individual contours from the experimental data yields the difference curve just above.

According to this method, the PM suspension was placed in the measurement cell of the microcalorimeter and heated from 20°C to 66°C (thick curve *a*, Fig. 1B). The sample was then cooled to 20°C and reheated to 82°C (thick curve *b*, Fig. 1B). The suspension was again cooled in the cell and the process was repeated for each transition in succession (see, thick curves *c* and *d* in Fig. 1B). If curve *b* is subtracted from curve *a*, we should get the initial portion of the curve shape and the temperature of the maximum (T_m) for the first transition (thin cuve *a*). Subtracting curve *c* from curve *b*, we obtain the initial portion of the curve and the T_m for the second transition. Repeating this procedure we get the data for each transition. The portion of each curve after the maximum was constructed for each transition by assuming symmetry relative to the corresponding T_m. It is interesting to note that only part of the DSC pretransition (second individual transition with T_m around 72°C) is reversible. To optimize the coincidence of the DSC experimental curve with the curve obtained by adding the individual transitions derived by successive

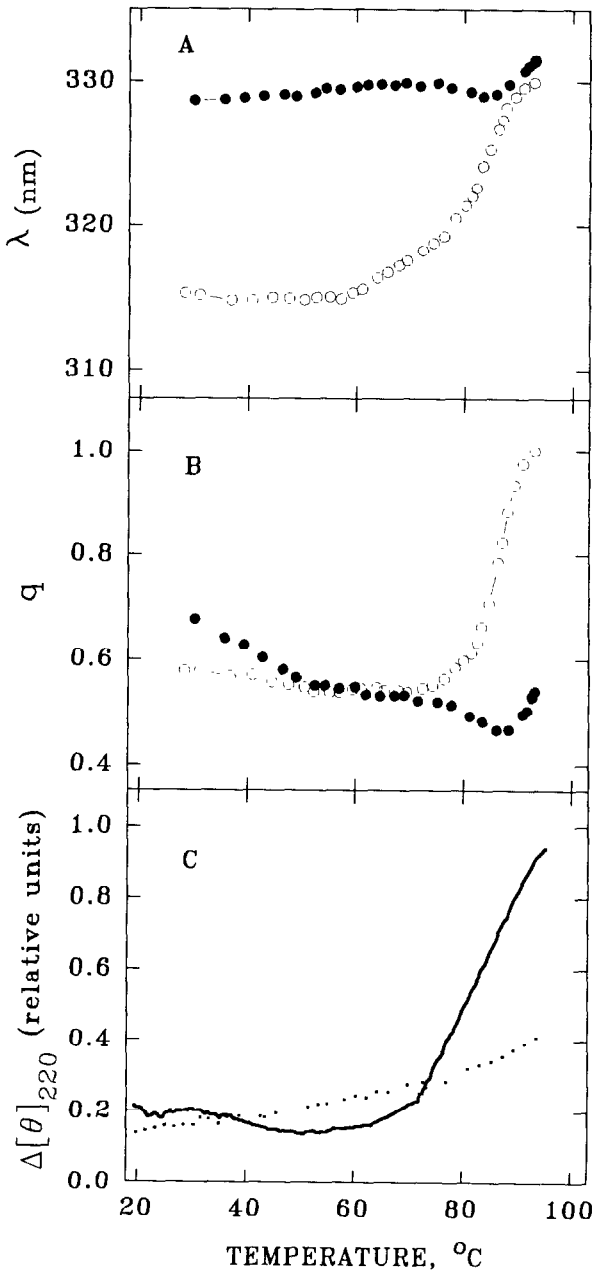

Fig. 2. Temperature dependence of the fluorescence and CD parameters of BR. (A) Fluorescence spectrum position, λ_{max}; (B) fluorescence quantum yield, q, and (C) temperature dependence of the ellipticity at 220 nm in relative units for PM suspension. In (A) and (B) hollow circles correspond to native PM and filled ones to PM previously kept at 85°C for 20 min (see text). In (C) the solid line corresponds to native PM and the dotted line to PM previously kept at 85°C for 20 min

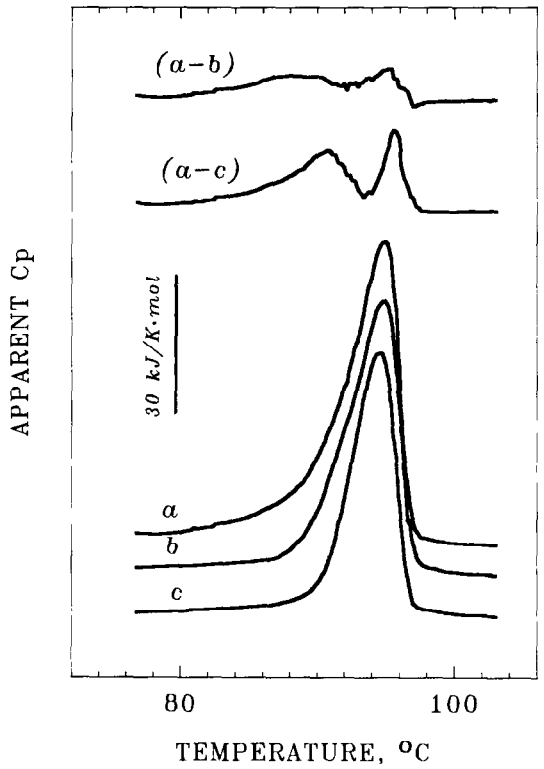

Fig. 3. Calorimetric profiles of PM in the temperature region of the main DSC transition. (*a*) Native PM; (*b*) PM after being kept at 85°C for 10 min; (*c*) PM after being kept at 85°C for 30 min. The difference curves are shown above.

annealing, only the intensity of the last transition had to be slightly altered. The result of the procedure and the errors of the curve decomposition are shown in Fig. 1C. Similar results were obtained when the annealing procedure was applied to a DSC scan of PM at 2°C/min, where only the T_m of the five resulting transitions increased somewhat, except for the second, reversible transition (results not shown).

Temperature-induced changes in BR were also followed by intrinsic fluorescence and CD spectroscopy. Fig. 2A,B shows the temperature dependence of the fluorescence parameters of BR in PM. Heating the suspension from 25°C to about 60°C did not significantly change the fluorescence spectrum position (Fig. 2A, hollow circles). A shift of the spectrum towards longer wavelengths begins above 60°C, where at least two distinct steps can be seen in the curve of λ_{max} vs. temperature. The main shift (of about 13 nm) occurs in the region from around 80°C to 90°C and is accompanied by a rise of the fluorescence relative quantum yield value (Fig. 2B, hollow circles). Nevertheless, both the spectrum position and the quantum yield did not show any co-operative transition after heating the sample at 85°C for 20 min (Fig. 2A,B, filled circles). Far-UV CD is also sensitive to BR denaturation, showing a sigmoidal ellipticity change centered at about 85°C (Fig. 2C, solid line). Again there is no evidence of any such transition when the PM suspension was kept previously at 85°C for 20 min (Fig. 2C, dotted line). This is not the case, however, in DSC. Thus, Fig. 3 shows that after having the sample standing at 85°C for 10 min, or even 30 min, a large portion of the main DSC transition still remains, becoming more symmetrical and comparatively sharper.

4. DISCUSSION

The annealing procedure leads to five transitions beneath the overall endotherm for PM. Although their thorough description in molecular terms would require a more extensive and systematic investigation, a preliminary tentative explanation can be given here. Shnyrov and Sukhomudrenko [25] have shown by deuterium exchange that with an increase in temperature some buried peptide groups are transferred to the protein surface in contact with water. This transfer reaches a maximum at about 62°C (the T_m of our first transition) and then decreases. Since CD shows no destruction of secondary structure below 70°C (Fig. 2C), the first transition may be related to some molecular rearrangement of the membrane leading to a less compact protein–lipid packing, thus allowing for the exposure of some residues. Jackson and Sturtevant [5] suggested that their reversible pretransition, which would correspond to our second reversible transition, was due to a structural reordering of the PM crystal lattice, an interpretation later supported by the X-ray studies of Hiraki et al. [26]. In our case, the changes in fluorescence within this temperature range would also suggest reversible changes in BR tertiary structure, indirectly affecting protein–protein interactions, with a resulting disorder of the crystal lattice. It is evident that no clear distinction can be drawn between our two first transitions, apart from their irreversible/reversible character. The heating of PM from 70°C to 90°C causes the irreversible formation of vesicles [7]. This process might be correlated with our third, irreversible transition, which occurs exactly within this temperature range.

Both our fourth and fifth transitions correspond to the main, asymmetric DSC peak, generally attributed to the BR irreversible denaturation. Nevertheless, the calorimetric and optical results give rise to some possible discrepancies concerning this interpretation. Firstly, CD and fluorescence transitions seem to be centered at about 85°C (a temperature comparable to the T_m of our fourth transition), which is about 10°C lower than the known T_m of the main DSC peak. Similar optical results on BR denaturation have been previously reported [5,11]. Secondly, when PM is kept at 85°C for 20 min (Fig. 2), re-heating the sample causes no co-operative change in CD or fluorescence, whereas after a similar treatment a clear, sharp peak still remains in the DSC re-heating scan, closely resembling our fifth transition

(Fig. 3). Therefore, it seems that a non-equilibrium state exists in the sample at around 85–90°C where the conformation of BR is already altered, but with a net DSC thermal transition still occurring in PM on a further increase in temperature. So our fourth transition might correspond to an irreversible conformational change in BR, as evidenced by the CD and fluorescence results. The remaining DSC transition in Fig. 3, which is analogous to our fifth annealing transition (Fig. 1C), would lead the protein to the final denatured state and could also be related to the complete destruction of the membrane, as reported by Shnyrov et al. [7], visualizing by electron microscopy after heating PM to about 100°C.

Finally, since BR denaturation is an irreversible, kinetically controlled process, as Galisteo and Sanchez-Ruiz [16] have recently proved, we heated all our samples at the same scan rate, i.e. 1°C/min, and at pH 7.5, where the two-state kinetic model does not apply [27]. To our knowledge this is the first, tentative attempt to further explain the rather complex DSC profile of PM, where only one protein (BR) exists, although with a unique, very well organized, bidimensional geometric array.

Acknowledgements. We are very grateful to Dr. L. Chekulaeva and Dr E. Padrós for providing the purple membranes, Drs. V. Akoev and V. Emelyanenko for excellent technical assistance, and Dr. V. Filimonov and Dr. E. Padrós for useful discussions. We also thank our colleague Dr J. Trout for revising the English text. This work was supported by Grants SAB92-0079 (V.L.Sh) and PB90-0876 (P.L.M.) from DGICYT of the Spanish Government.

REFERENCES

[1] Stoeckenius, W. and Bogomolni, R.A. (1982) Annu. Rev. Biochem. 52, 587–616.
[2] Khorana, H.G (1988) J. Biol. Chem. 263, 7439–7442.
[3] Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) J. Mol. Biol. 213, 899–929.
[4] Kates, M., Kushawaha, S.C and Sprott, G.D. (1982) Methods Enzymol. 88, 98–111.
[5] Jackson, M.B. and Sturtevant, J M. (1978) Biochemistry 17, 911–915.
[6] Lazarev, Yu.A. and Shnyrov, V.L. (1979) Bioorg. Khim. (Moscow) 5, 105–112.
[7] Shnyrov, V.L., Tarakhovsky, Y.S. and Borovyagin, V.L. (1981) Bioorg. Khim. (Moscow) 7, 1054–1059
[8] Shnyrov, V.L., Zakis, V.I. and Borovyagin, V.L. (1984) Biol. Membranes (Moscow) 4, 349–355.
[9] Brouilette, C.G., Muccio, D.D. and Finney, T.K. (1987) Biochemistry 26, 7431–7438.
[10] Brouilette, C.G., McMichens, R.B., Stern, L.J. and Khorana, H.G. (1989) Proteins: Struct. Funct. Genet. 5, 38–46.
[11] Cladera, J., Galisteo, M.L., Duñach, M., Mateo, P.L. and Padrós, E. (1988) Biochim. Biophys. Acta 943, 148–156.
[12] Cladera, J., Galisteo, M.L., Sabes, M., Mateo, P.L. and Padrós, E. (1992) Eur. J. Biochem. 207, 581–585
[13] Maglova, L., Guleva, D., Chekulaeva, L. and Atanasov, B (1990) Biochim. Biophys. Acta 1017, 217–220.
[14] Kresheck, G.C., Lin, C.T., Williamson, L.N., Mason, W.R., Jang, D.J. and El-Sayed, M.A. (1990) Photochem. Photobiol. 7, 289–302.
[15] Kahn, T.W., Sturtevant, J.M. and Engelman, D M. (1992) Biochemistry 31, 8829–8839.
[16] Galisteo, M.L. and Sanchez-Ruiz, J.M. (1993) Eur. Biophys. J. (in press).
[17] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[18] Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–327.
[19] Privalov, P L. (1980) Pure Appl. Chem. 52, 479–497.
[20] Teale, F.W J. and Weber, G. (1957) Biochem. J. 65, 476–482.
[21] Shnyrov, V.L., Zhadan, G.G. and Akoev, I.G. (1984) Bioelectromagnetics 5, 411–418.
[22] Shnyrov, V.L. and Berman, A L. (1988) Biomed. Biochim. Acta 4–5, 355–362.
[23] Permyakov, E.A., Shnyrov, V.L., Kalinichenko, L.P., Kuchar, A., Reyzer, I.L and Berliner, L.J. (1991) J. Prot. Chem 10, 577–584.
[24] Levitsky, D.I., Shnyrov, V L., Khvorov, N.V, Bukatina, A.E., Vedenkina, N.S., Permyakov, E.A., Nikolaeva, O.P. and Poglazov, B.F (1992) Eur. J. Biochem. 209, 829–835.
[25] Shnyrov, V.L. and Sukhomudrenko, A.G. (1986) Biophys. Chem. 24, 1–4.
[26] Hiraki, K., Hamanaka, T., Mitsui, T and Kito, Y. (1981) Biochim. Biophys. Acta 647, 18–28.
[27] Galisteo, M.L. (1990) Ph.D Thesis, University of Granada (Spain).